Storage and Maintaining Activity of Ribulose Bisphosphate Carboxylase/Oxygenase

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ABSTRACT

Purified ribulose-1,5-bisphosphate carboxylase/oxygenase in 50% saturated (NH4)2SO4 was stable when frozen as small beads in liquid nitrogen and stored at −80°C. When stored as a slurry at 4°C most of the activity was lost within four weeks. This loss was due not only to enzyme polymerization. Activity in old preparations purified from spinach leaves, but not tobacco or tomato leaves, can be restored to the level of newly purified enzyme after storage at 4°C by treatment with 50 to 100 millimolar dithiothreitol for several hours followed by dialysis against buffer and 1 millimolar dithiothreitol before CO2 and Mg2+ activation and assay. Some enzyme oligomers that had been formed were not converted back to native enzyme by treatment with 100 millimolar dithiothreitol.

The purified enzyme contained about 2 gram-atoms iron per mole enzyme that could not be removed by chelating agents. When the enzyme was incubated with 100 millimolar dithiothreitol and exposed to O2, a purple dithiothreitol-iron complex was formed which could be removed by dialysis. The activities of ribulose-1,5-bisphosphate carboxylase and oxygenase were not altered by reducing the iron content to 0.7 mole per mole enzyme by treatment with dithiothreitol followed by exhaustive dialysis against iron free buffer.

Purified d-ribulose-P2, carboxylase/oxygenase (E.C. 4.1.1.39) preparations from spinach leaves, stored in 50% saturated (NH4)2SO4 at 4°C lose 60 to 80% of their activities within 3–6 weeks after purification (9, 14), and after 6 weeks the preparations have generally been abandoned. Even though this protein can be prepared pure in gram quantities, there is a need for improved techniques of storage and of stabilization of its activity. The activity of the freshly prepared enzyme is largely affected by DTT (20), suggesting that one of the changes occurring during storage may be an oxidation of sulfhydryl groups. This paper describes two ways to reduce the loss of activity during storage. One is a rapid freezing into small beads in liquid N2 and storage at −80°C; the other is a treatment of preparations that had been stored at 4°C with 100 mM DTT for several hours at room temperature to restore activity.

During the treatment with DTT, a purple color developed in the enzyme preparations which has been attributed to an iron-DTT complex. This color was particularly evident with enzyme preparations containing more than 10 mg protein/ml. The iron content of ribulose-P2 carboxylase/oxygenase has been reported to be 0.13 or 0.25 mol/mol enzyme (3, 21), but the amounts we have found are somewhat higher. The purified enzyme has been reported to contain about 2 g-atoms of copper per mol of enzyme (3, 10, 21), and one report on the crystalline enzyme from spinach leaves indicated 0.15 g-atom of iron per mol and only 0.05 g-atom of copper (8). From these reports, it is assumed that neither copper nor iron are involved with catalysis but continued uncertainty about the mechanism of the oxygenase reaction necessitates continued evaluation of the metal ion content of the enzyme.

MATERIALS AND METHODS

Materials. Most of our spinach (Spinacia oleracea c.v. Virlofly 99) was grown under short day conditions in a greenhouse, but similar results were obtained with field grown plants. Ribulose-P2 carboxylase/oxygenase was purified from spinach leaves by (NH4)2SO4 precipitation, gel filtration, and DEAE chromatography (16). Different preparations of the purified enzyme had initial specific activities of 1.0 to 2.2 μmol CO2/mg protein/min when assayed at 30°C in 10 mM NaHCO3. Preparations have been routinely stored in this laboratory as a 50% (NH4)2SO4 slurry.

Ribulose-P2 was synthesized enzymically from ribose-5-phosphate (7). DTT was obtained from Sigma and (NH4)2SO4 (ultrapure grade) from Schwarz Mann, Spring Valley, NY. [14C]-NaHCO3 was from Amersham-Searle, Arlington Heights, IL.

The dialysis buffer was 50 mM N,N-bis(2-hydroxyethyl)glycine (Sigma, Bicine) with 0.4 mM EDTA, adjusted to pH 8.0 at 22°C with KOH. A 0.2 mM DTT solution was prepared using the dialysis buffer which had been flushed with N2 immediately before use. This solution was added to the enzyme preparation to provide a designated final concentration of DTT.

Freezing in Liquid Nitrogen and Storage at −80°C. The purified enzyme as a slurry in 50% (NH4)2SO4 was dropped slowly (about 2 drops/s) from a Pasteur pipette or separatory funnel into a large flask of liquid N2 so that the individual drops froze rapidly into small beads. The liquid N2 was then decanted and the contents stored in a −80°C freezer. The beads may be poured out of the cold storage flask without thawing the whole preparation. This method has been used for the storage of other enzymes (22). Before assaying, the enzyme was thawed at room temperature and dissolved in dialysis buffer with 10 mM DTT. In some cases the thawed enzyme was adjusted to 10 mM DTT by adding 0.2 mM DTT and allowed to stand for 5–6 h. The protein in this 50% (NH4)2SO4 slurry was collected by centrifugation at 27,000g at 4°C for 20 min. Then the precipitated enzyme was dissolved in dialysis buffer containing 1 mM DTT. After dialysis, the enzyme was kept at room temperature in buffer with 1 mM DTT.

Determination of Iron. Solutions were prepared with glass distilled H2O that had been passed over Chelex 100. The iron content...
of Bicine and ribulose-P$_2$ solutions was less than 0.1 nmol per
μmol of reagent. Ultra pure reagents, such as Mg(NO$_3$)$_2$, KOH,
HCl and NaHCO$_3$ were from the Vrenton Alfa Corp., Danvers,
MA. The iron content of the enzyme was measured by wet ashing
with iron-free H$_2$SO$_4$, HNO$_3$ and HClO$_4$ after which the total iron
was measured by complexing with bathophenanthroline and ex-
tracted into isoamyal alcohol (19). This colorimetric method was
linear up to at least 1.5 μg iron.

**Treatment and Analysis of the Enzyme with 100 mM DTT.**
Enzyme at 20–30 mg protein per ml was diluted 1:1 with freshly
prepared solutions of DTT that provide the desired final DTT
concentrations of 0 to 100 mM. These were stored in a closed vial
with no air space for 5–6 h or overnight. Before assay of the
enzyme activity the DTT concentration was reduced by dialysis
for several h against buffer containing 1 mM DTT, or by dilution
of the concentrated protein with dialysis buffer. Ribulose-P$_2$
carboxylase/oxygenase was activated for at least 15
min before assay by preincubation in 10 mM NaHCO$_3$ and 20 mM
MgCl$_2$ (11), and assayed as described previously (12, 13). Protein
was estimated from its A at 280 nm (14). Since the oxidized form
of DTT absorbs strongly at 280 nm (4), it cannot be present for
this determination. Ribulose-P$_2$ carboxylase/oxygenase was ana-
yzed on 7.5% acrylamide gels (6), and the protein detected by
staining overnight in Coomassie Brilliant Blue.

**RESULTS**

**Stability of Enzyme Stored at −80 C.** Different enzyme prep-
arrations from spinach leaves were precipitated in 50% (NH$_4$)$_2$SO$_4$,
the suspension quickly frozen into small beads in liquid N$_2$, and
the beads stored at −80 C. Enzyme preparations stored for 6
months or longer retained 70 to 90% of their activity. Treatment of
these stored preparations afterwards with 25 or 50 mM DTT, as
described in a later section, increased the activity to about 85–95%
of the original activity. When an enzyme preparation was dialyzed
to remove most of the (NH$_4$)$_2$SO$_4$ before storage at −80 C it lost
about 65% of its original activity during storage, but addition of
10 to 50 mM DTT restored the activity to near its original value.
At present we freeze purified enzyme preparations in 50% (NH$_4$)$_2$SO$_4$
as beads in liquid N$_2$, store them at −80 C, and incubate them with 10 or 20 mM DTT after thawing. The purified enzyme
from *Rhodospirillum rubrum* has also been stored at −60
C with 20% glycerol (17), but in the above procedure no glycerol
is needed.

**Restoring Activity of Preparations with Dithiothreitol.** 1 mM
DTT has been used routinely in preparations of ribulose-P$_2$
carboxylase/oxygenase to protect its many (about 96) sulfhydryl
groups (18). When a preparation was stored in a slurry of 50%
saturated (NH$_4$)$_2$SO$_4$ at 4 C for 1 month or longer, it had lost a
great deal of its activity when redissolved in 1 or 10 mM DTT
(Table I). When these 1- to 3-month-old preparations were pre-
treated with either 50 or 100 mM DTT for several h before assay,
full activity was restored. Longer storage periods in 50% saturated
(NH$_4$)$_2$SO$_4$ have not been tested except for some very old prepa-
rations that had been stored at 4 C. In these, about 25% of the
original activity was restored by 50 or 100 mM DTT (Table I).
Treatment of freshly prepared enzyme preparations with 50 or 100
mM DTT had no effect on the specific activity.

Previous studies (9, 10) have shown that during storage in 50%
(NH$_4$)$_2$SO$_4$, the enzyme forms high mol wt aggregates that can be
separated on polyacrylamide gels or sucrose gradients. One en-
zyme preparation which had been stored in 50% (NH$_4$)$_2$SO$_4$ for 4
years had 4 major bands of higher mol wt than the native
carboxylase, but the major portion of this preparation still mi-
grated on polyacrylamide gels similarly to the monomer or freshly
prepared enzyme. The higher mol wt polymers are not present in
freshly prepared enzyme samples to any appreciable extent. Treat-
ment of this old enzyme with 100 mM DTT did not remove the
high mol wt species. Thus, the partial reactivation of this old
preparation by 100 mM DTT may be attributed to a change in the
monomeric enzyme rather than a depolymerization of the aggre-
gates. Determination of the specific activity, if it had been calcu-
lated on the basis of the amount of monomer alone, would have
been somewhat higher, but even taking this into account the specific activity was not increased to the original activity of the
freshly prepared enzyme (data not shown).

The enzyme preparation that was assayed after 1 and 3 months
storage at 4 C in 50% (NH$_4$)$_2$SO$_4$ and used for data in Table I,
chromatographed on a Sepharose 4B column as a single protein
band. Its specific activity before and after filtration did not change
(data not shown). Significant amounts of polymers had not yet
been produced, even though the activity of the enzyme had
decreased over 60%. The activity of this preparation was restored to
0.8 to 0.9 μmol CO$_2$/mg protein·min by 50 or 100 mM DTT
treatment after passage through the Sepharose 4B column. Thus,
our limited studies on the reactivation by very high concentrations
of DTT indicate polymerization and depolymerization cannot
explain the loss and restoration of activity. It is possible that
during storage in (NH$_4$)$_2$SO$_4$, the enzyme forms mixed disulfides
with β-mercaptoethanol left in solution from the final purification
step. In this case, the high concentration of DTT may serve to
convert these enzyme bound mixed disulfides back to the free
thiols, as suggested for a mechanism of activation for carbonic
anhydrase (5).

**Table I. Effect of DTT Concentration on Ribulose-P$_2$
carboxylase/Oxygenase Activity**

| DTT Concentration | Ribulose-P$_2$ Carboxylase | Ribulose-P$_2$ Oxy-
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>mM</td>
<td>μmol/mg protein·min</td>
<td>genease</td>
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<tr>
<td>0</td>
<td>1.6</td>
<td>0.18</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>0.16</td>
</tr>
<tr>
<td>50</td>
<td>1.5</td>
<td>0.16</td>
</tr>
<tr>
<td>100</td>
<td>1.7</td>
<td>0.19</td>
</tr>
<tr>
<td>Preparation stored for 2 weeks*</td>
<td>1.1</td>
<td>0.12</td>
</tr>
<tr>
<td>0</td>
<td>1.4</td>
<td>0.16</td>
</tr>
<tr>
<td>Preparation stored for 1 month*</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>Preparation stored for 3 months*</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>Preparation stored for 4 years</td>
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<tr>
<td>0</td>
<td>0.09</td>
<td>0.01</td>
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<tr>
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<td>0.03</td>
</tr>
<tr>
<td>50</td>
<td>0.3</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* Activity of this preparation when freshly prepared was 1.1 μmol CO$_2$/
mg protein·min.
shown in Figure 1. Bentle and Lardy (1) have associated this color with a possible iron-DTT complex. Removal of the O₂ from the ribulose-P₂ carboxylase/oxygenase preparations by flushing with N₂ prevented the appearance of this color when DTT was added. Solutions of FeSO₄ and DTT have similar absorption spectra in the presence of O₂ (data not shown).

Pure preparations of ribulose-P₂ carboxylase/oxygenase were prepared from spinach leaves with reagents of lowest iron content available and then dialyzed extensively against quantitatively iron-free Bicine in the presence of up to 5 mM EDTA. These enzyme preparations contained about 2 g-atoms/iron/mol enzyme by chemical analyses (Table II). Dialysis of the enzyme overnight against 100 mM DTT lowered the iron content from 2.0 to 0.7 g-atom/mol enzyme without any change in the specific activity of either the carboxylase or oxygenase. In this case the controls were the enzyme pretreated without DTT. The treatment of ribulose-P₂ carboxylase with a high concentration of a reducing agent, such as DTT, seems to result in the removal of iron. This is the usual case where the more oxidized state of a transition metal is bound very tightly and reduction allows for the removal of the metal. Considering the low stoichiometry of the bound iron in the enzyme and the fact that removal of most of the iron had no effect on either carboxylase or oxygenase activity, we agree with others (3, 8, 21) that bound iron is unlikely to have a catalytic role in the mechanism of catalysis.

Acknowledgments.—Dr. Orme-Johnson suggested storage by the liquid N₂ freezing method and helped with the iron determinations. Dr. Henry Lardy predicted that the purple color formed upon adding DTT might be due to an iron-DTT complex.

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